RanBP3 Regulates Melanoma Cell Proliferation via Selective Control of Nuclear Export

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Chromosome region maintenance 1-mediated nucleocytoplasmic transport has been shown as a potential anticancer target in various malignancies. However, the role of the most characterized chromosome region maintenance 1 cofactor ran binding protein 3 (RanBP3) in cancer cell biology has never been investigated. Utilizing a loss-of-function experimental setting in a vast collection of genetically varied melanoma cell lines, we observed the requirement of RanBP3 in melanoma cell proliferation and survival. Mechanistically, we suggest the reinstatement of transforming growth factor-β (TGF-β)-Smad2/3-$p21^{Cip1}$ tumor-suppressor axis as part of the RanBP3 silencing-associated antiproliferative program. Employing extensive nuclear export sequence analyses and immunofluorescence-based protein localization studies, we further present evidence suggesting the requirement of RanBP3 function for the nuclear exit of the weak nuclear export sequence-harboring extracellular signal-regulated kinase protein, although it is dispensable for general CRM1-mediated nuclear export of strong nuclear export sequence-harboring cargoes. Rendering mechanistic support to RanBP3 silencing-mediated apoptosis, consequent to extracellular signal-regulated kinase nuclear entrapment, we observed increased levels of cytoplasmically restricted nonphosphorylated/active proapoptotic Bcl-2-antagonist of cell death (BAD) protein. Last, we present evidence suggesting the frequently activated mitogen-activated protein kinase signaling in melanoma as a potential founding basis for a deregulated post-translational control of RanBP3 activity. Collectively, the presented data suggest RanBP3 as a potential target for therapeutic intervention in human melanoma.

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INTRODUCTION

MEK-ERK signaling is central to melanoma cell biology (Flaherty et al., 2012). Even so, the approaches directly targeting this constitutively activated pathway through small molecule inhibitors have accomplished encouraging initial clinical responses, their long-term success has been confounded by an expansive array of acquired resistance mechanisms (Solit and Rosen, 2014).

Although cellular signaling pathways and the molecular perturbations in their component proteins have frequently been associated with various aspects of cancer cell biology (Hanahan and Weinberg, 2011), the altered protein subcellular distribution, especially a cytoplasmic buildup of various tumor suppressor proteins, has started to emerge as another significant mechanism in cancer pathogenesis and therapeutic resistance (Cagnol and Chambard, 2010; Jiao et al., 2008; Kau et al., 2004; Turner and Sullivan, 2008). For example, the aberrant cellular distribution of inhibitory cell cycle regulators (p53, $p21^{Cip1}$, and $p27^{Kip1}$), transcription factors (FOXO and NF-κB), and tumor suppressors (INI1/hSNF5 and BRCA1) has been associated with cancer progression (Kau et al., 2004).

With chromosome region maintenance 1 (CRM1) being the core component of the nuclear export machinery, a gene frequently overexpressed in cancer cells (Huang et al., 2009; van der Watt et al., 2009), including melanoma (Pathria et al., 2012), and a candidate whose expression is associated with poor disease prognosis (Noske et al., 2008; Shen et al., 2009), most nuclear export inhibition strategies have primarily focused on developing novel CRM1 inhibitors (Turner et al., 2014). These efforts have translated into highly efficacious and relatively non-toxic Selective Inhibitors of Nuclear Export (SINE) class of CRM1 inhibitors that have recently made their way into the clinical trials (Gerecitano, 2014).

Ran binding protein 3 (RanBP3), a protein characterized as a cofactor for CRM1-mediated nuclear export, has been shown to be involved in the early steps of CRM1-mediated

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Abbreviations: BAD, Bcl-2-antagonist of cell death; CRM1, chromosome region maintenance 1; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase 1/2; NES, nuclear export sequence; MAKP, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; RanBP3, ran binding protein 3; siRNA, small interfering RNA; TGF-β, transforming growth factor-β

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export, where it helps stabilize the ternary complex between RanGTP, CRM1, and the export cargo. Furthermore, a recent study reports RanBP3 as a crucial player downstream of the oncogenic mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase signaling pathways, thus possibly linking aberrant oncogenic signaling with deregulated nuclear export activity (Yoon et al., 2008).

Although the known functionality as a CRM1 cofactor and the associations with major oncogenic signaling pathways would argue for a potential role of RanBP3 in cancer biology, surprisingly, never has this proposition been systematically investigated. Therefore, in this study we tested the requirement of RanBP3 function in melanoma biology and evaluated its candidacy as a therapeutic target.

RESULTS

RanBP3 is required for melanoma cell proliferation and survival

To investigate the role of RanBP3 in melanoma cell biology, we adopted a loss-of-function approach. RanBP3 silencing utilizing small interfering RNA (siRNAs) suppressed melanoma cell proliferation (Figure 1a). Furthermore, RanBP3 silencing in an extended panel of genetically heterogeneous—encompassing virtually every major melanoma-associated molecular alteration (mut-*BRAF*, mut-*NRAS*, mut-*NF-1*, mut-/del-*CDKN2A* (p16INK4a), mut-/del-*PTEN*, and mut-*TP53*; Supplementary Table S1 online)—melanoma cell lines similarly compromised proliferation (Figure 1b and Supplementary Figure S1 online). An increase in annexinV and annexinV + propidium iodide positive cell fraction on RanBP3 silencing demonstrated apoptotic induction in all the tested cell lines (Figure 1c). Further indication of caspase-3 cleavage and pan-caspase inhibitor-mediated alleviation of cell death corroborated its apoptotic nature (Supplementary Figure S2a and b online). Notably, WM983B-BR melanoma cells that are resistant to BRAF-inhibitor PLX-4032 (cultured in growth medium comprising PLX-4032) also exhibited apoptotic cell death on RanBP3 silencing (Figure 1d). However, RanBP3 silencing in primary human skin fibroblasts failed to elicit apoptotic cell death (Figure 1e). Further underscoring the differential dependence of melanoma cells on RanBP3 function, RanBP3 silencing in telomerase reverse transcriptase-immortalized (Pmel/hTERT/CDK4(R24C)/p53DD) nontransformed primary human melanocyte lines genetically engineered to ectopically express either $BRAF^{V600E}$ (referred to as HMEL-B) or $NRAS^{G12D}$ (referred to as HMEL-N) did not induce significant cell death (Supplementary Figure S2c). These results underscore RanBP3's requirement in melanoma cell proliferation and survival, although it is dispensable in a noncancerous setting.

Interestingly, a closer look at MeWo cells depicted morphological features typical of senescence (Supplementary Figure S3a online). Loss of light refractivity, enlargement, and increased vacuolization were observed in a high percentage of cells. RanBP3 silencing resulted in a high percentage of cells staining positive for the established senescence marker, β-galactosidase, and suppression of phospho-retinoblastoma levels (Supplementary Figure S3b and c).

Altogether, these data suggest RanBP3 as a regulator of melanoma cell proliferation and survival.

RanBP3 negatively modulates the TGF-β-Smad2/3-$p21^{Cip1}$ growth suppressor axis

RanBP3 has previously been reported as a nuclear export mediator of Smad2/3 and, thus, a negative modulator of the TGF-β-regulated $p21^{Cip1}$/$p15^{INK4b}$-mediated tumor suppressive program in primary human keratinocytes (Dai et al., 2009). In line with this report, we also observed the requirement of RanBP3 function for Smad2/3 nuclear export in a melanoma setting (Supplementary Figure S4 online). Consistently, in the melanoma cells, RanBP3 knockdown also triggered an induction of $p21^{Cip1}$ levels, an effect that was further augmented by the presence of TGF-β (Figure 2a). Interestingly, however, no change in $p15^{INK4b}$ protein levels was observed (Figure 2a). Furthermore, an increase in the G1 cell cycle fraction (Supplementary Figure S5 online) supported the increased $p21^{Cip1}$ and suppressed phospho-retinoblastoma levels (Supplementary Figure S3c). Importantly, a concomitant $p21^{Cip1}$ silencing, partially, yet significantly, relieved the antiproliferative action of RanBP3 silencing (Figure 2b and c). Furthermore, transforming growth-factor receptor pathway inhibition partially overcame $p21^{Cip1}$ induction and the antiproliferative activity associated with RanBP3 silencing (Figure 2d and e).

These data demonstrate a conserved negative association between RanBP3 and the TGF-β-Smad2/3-$p21^{Cip1}$ growth suppressive axis in a transformed cellular context.

RanBP3 and CRM1-mediated nuclear export

Although RanBP3 is frequently referred to as a cofactor for CRM1-mediated nuclear export, a systematic interrogation of its requirement in the nuclear exit of established CRM1 substrates has never been undertaken. Furthermore, to gain a better understanding of the additional mechanisms underlying the antiproliferative and apoptotic outcome associated with RanBP3 targeting, we undertook a detailed functional assessment of RanBP3 involvement in the nuclear export of three (Survivin, p53, and MEK1) previously established CRM1 substrates (Henderson and Eleftheriou, 2000). Notably, survivin and p53 were additionally implicated as the mechanistic basis for CRM1 inhibition-mediated apoptosis (Pathria et al., 2012). As reported previously (Pathria et al., 2012), Leptomycin B-mediated CRM1 inhibition triggered survivin nuclear entrapment (Figure 3a). In contrast, RanBP3 silencing failed to elicit a change in the survivin cellular distribution (Figure 3b). As shown previously (Chan et al., 2010), after its nuclear entrapment, the suppression of survivin levels was evident (Figure 3c), whereas, consistent with its failure to trigger survivin nuclear entrapment, RanBP3 silencing also failed to mitigate its expression levels (Figure 3c).

p53 is exported in a CRM1-dependent manner, with CRM1 inhibition triggering its nuclear entrapment (Freedman and Levine, 1998), overcoming its cytoplasmic proteasomal degradation, and thus, inducing its protein levels (Figure 3d). Again, in contrast to CRM1 inhibition, RanBP3 silencing failed to influence the p53 subcellular distribution or protein expression levels (Figure 3e). In line with its dispensability for the nuclear export of survivin and p53, MEK1 nuclear export, while relying on CRM1 function (Figure 3f), showed indifference to RanBP3 function (Figure 3g).

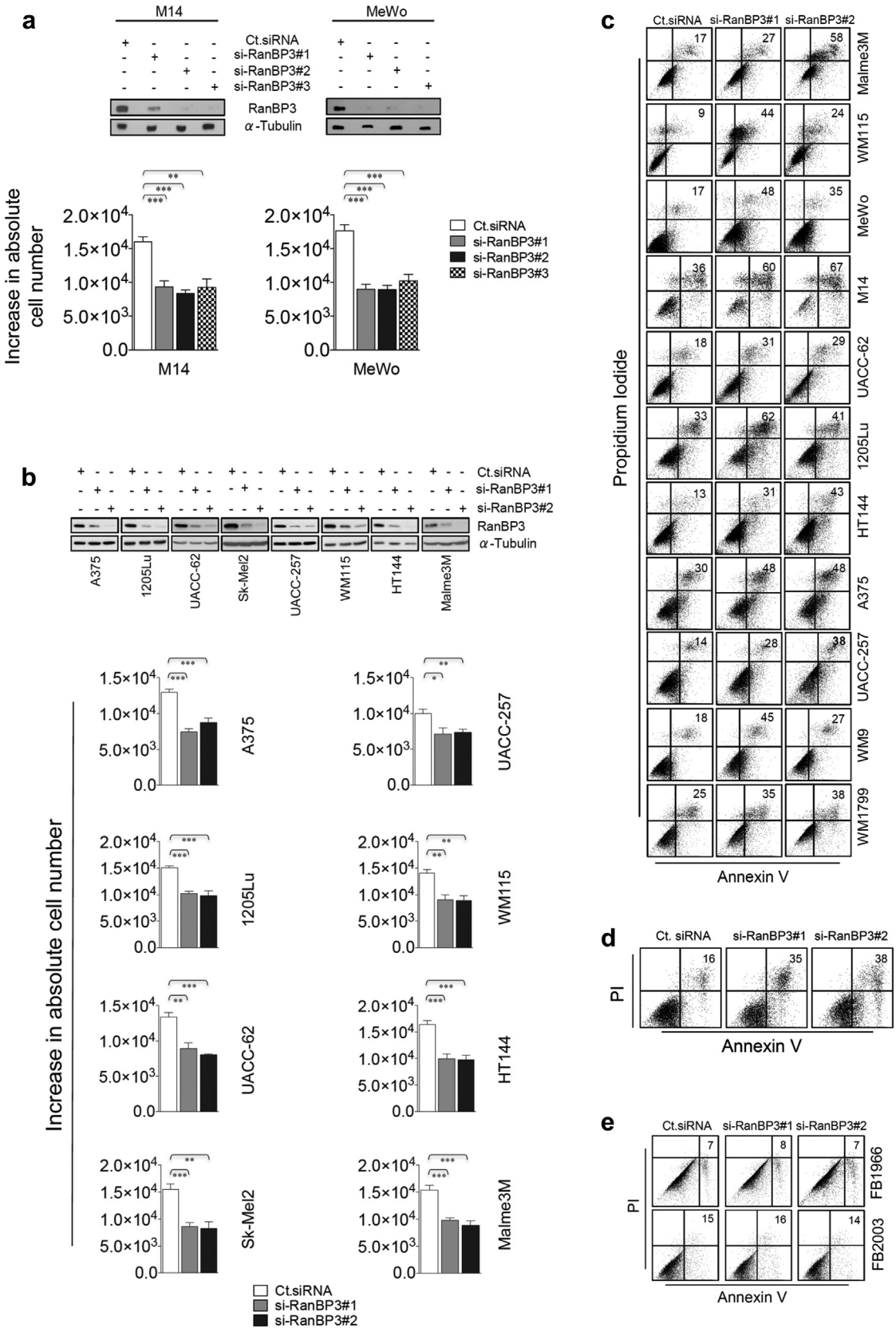

Figure 1. RanBP3 is required for melanoma cell proliferation and survival. (**a**) Upper panel: M14 and MeWo cells were transfected with control small interfering RNA (Ct.siRNA) or the indicated siRNA-RanBP3 (si-RanBP3#1, si-RanBP3#2, or si-RanBP3#3) for 72 hours followed by immunoblotting for RanBP3. Lower panel: M14 and MeWo cells were treated as indicated. The increase in the absolute cell number (*y*-axis) was determined by subtracting the cell number at the time of seeding from the cell number 96 hours after siRNA transfection. Error bars represent ± SD from triplicates. (**b**) Upper panel: Indicated melanoma cell lines were treated with Ct.siRNA or si-RanBP3 (#1 or #2) followed by the assessment of RanBP3 levels by immunoblotting. Lower panel: Indicated

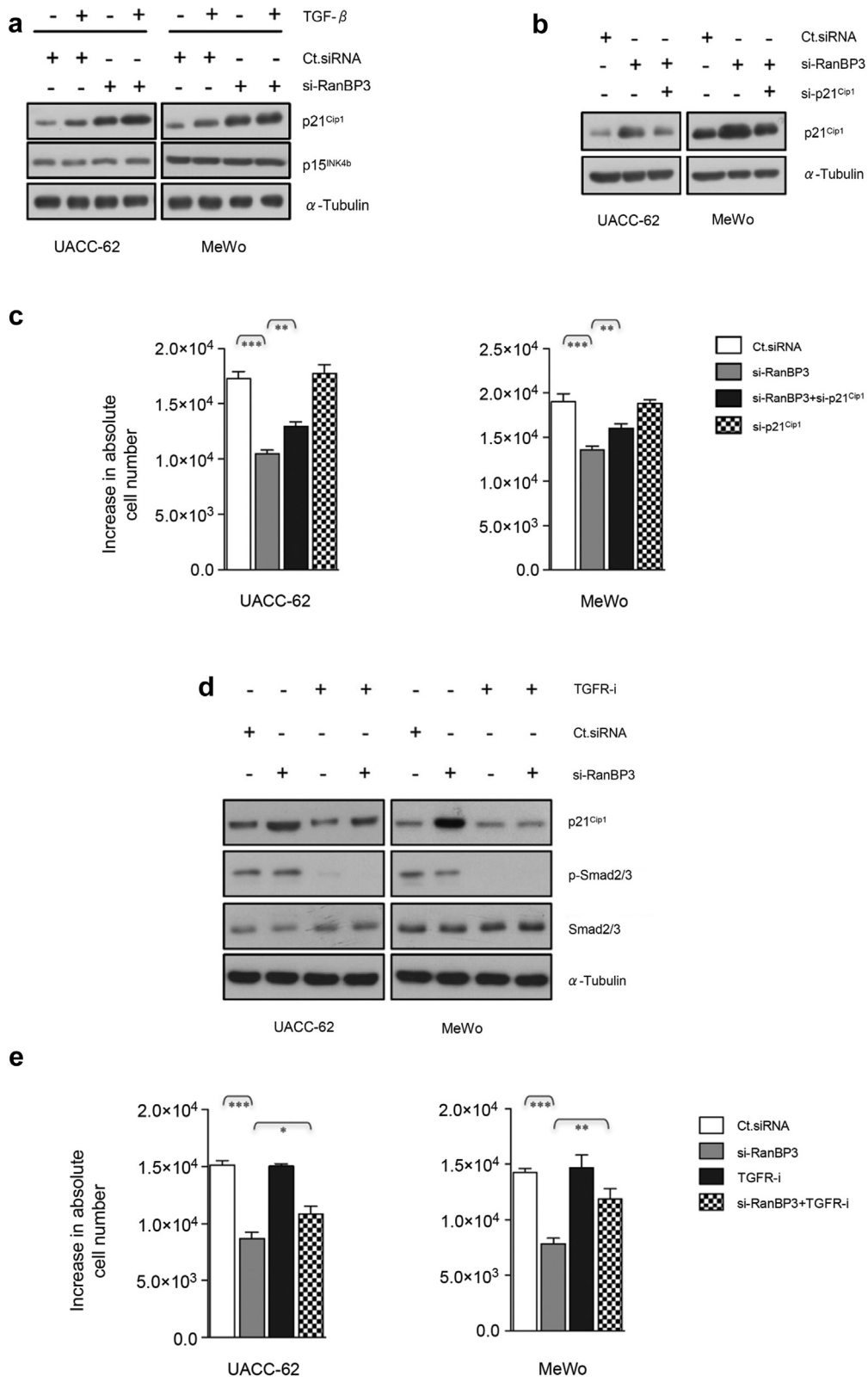

Figure 2. RanBP3 regulates TGF-β-p21^{Cip1} signaling axis. (**a**) UACC-62 and MeWo cells were treated as indicated for 72 hours followed by the assessment of the p21^{Cip1} and p15 levels by immunoblotting; 2 ng/ml of TGF-β was used. (**b**) UACC-62 and MeWo cells were treated as indicated for 72 hours followed by immunoblotting for the assessment of p21^{Cip1}. (**c**) UACC-62 and MeWo cells were treated as indicated for 96 hours. The *y*-axis depicts the increase in the absolute cell number. Error bars indicate ±SD from triplicate values. (**d**) Immunoblotting for p21^{Cip1}, phospho(p)-Smad2/3, and Smad2/3 in UACC-62 and MeWo cells on indicated treatments for 96 hours. (**e**) UACC-62 and MeWo cells were treated as indicated for 96 hours. The *y*-axis depicts the increase in the absolute cell number. Error bars indicate ±SD from triplicate values. The TGF-β inhibitor SB-431542 was used at a concentration of 5 nM. TGF-β inhibitor treatment was initiated 48 hours after siRNA treatment. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ (*t*-test). RanBP3, ran binding protein 3; siRNA, small interfering RNA; TGF-β, transforming growth factor-β.

melanoma cell lines were treated with Ct.siRNA or si-RanBP3 (#1 or #2). The increase in the absolute cell number (*y*-axis) was determined 96 hours after siRNA transfection. Error bars indicate ±SD from triplicates. (**c**) Indicated melanoma cell lines were treated with Ct.siRNA or si-RanBP3 (#1 or #2) for 96 hours followed by annexinV and propidium iodide (PI) staining. Numbers on the top-right quadrant indicate the proportion of annexinV-positive (early apoptosis) + annexinV and PI-positive (late apoptosis) cells. (**d**) BRAF-i-resistant WM983B-BR cells were treated as indicated followed by annexinV and PI-based apoptotic assessment. (**e**) FB2003 and FB1966 fibroblast cell lines were treated as indicated followed by annexinV and PI staining. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ (*t*-test). RanBP3, ran binding protein 3; siRNA, small interfering RNA.

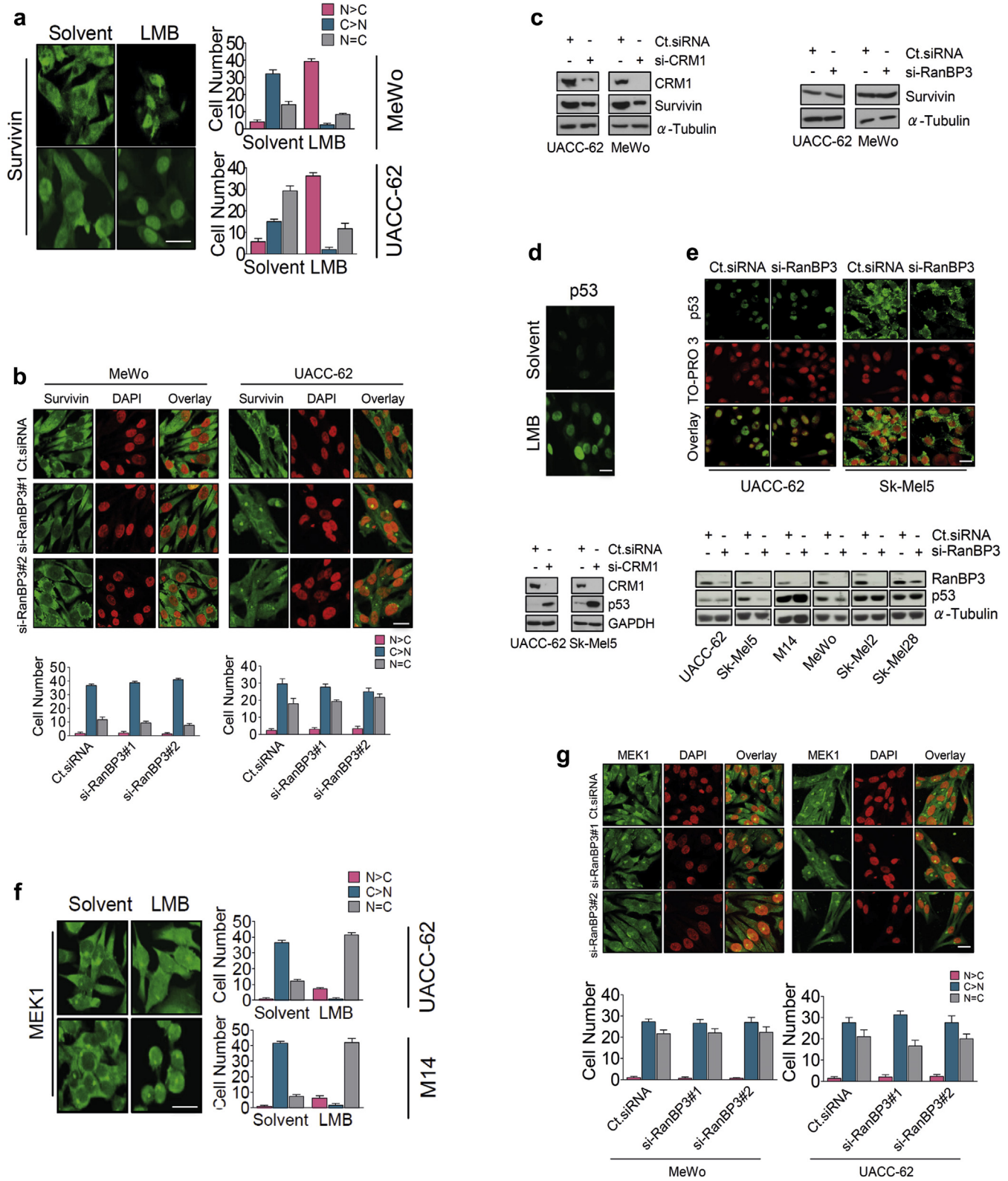

Figure 3. RanBP3 in CRM1-mediated nuclear export. (**a**) Left: Immunofluorescence-based staining for survivin in the indicated cell lines on 6 hours' LMB (4 nM) treatment. Right: Quantification of survivin cellular distribution. 50 cells were randomly scored and plotted (*y*-axis) as cell number with predominant nuclear ($N > C$), predominant cytoplasmic ($C > N$), or equal nuclear and cytoplasmic ($N = C$) survivin distribution. Error bars indicate ±SD from three random scorings. Scale bar = 20 μm. (**b**) Top: Immunofluorescence-based staining for survivin (left panel) in the indicated cell lines on 72 hours' treatment with Ct.siRNA or si-RanBP3 (#1 or #2). Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; middle panel). Overlay of survivin and nuclear stain (right panel). Bottom: Corresponding quantification. Scale bar = 20 μm. (**c**) UACC-62 and MeWo cells were transfected with si-CRM1 (left panel) or si-RanBP3#1 (right panel) for 72 hours followed by immunoblotting for survivin. (**d**) Top: Immunofluorescent staining for p53 in UACC-62 cells on 6 hours of LMB (4 nM) treatment. Scale bar = 20 μm. Bottom: UACC-62 and Sk-Mel5 cells were transfected with Ct.siRNA or si-CRM1. After 72 hours, p53 levels were assessed by

Previously, the work of Englmeier et al. (2001) suggested the requirement of RanBP3 in the CRM1-mediated nuclear export of the proteins harboring a weak, but not a strong, nuclear export sequence (NES). Our own studies (Figure 3) refuting the requirement of RanBP3 in the export of survivin, p53, and MEK1 further corroborate this suggestion. To experimentally validate this proposition, we undertook systematic sequence alignment studies to establish the NES consensus. Utilizing previously validated (Henderson and Eleftheriou, 2000) and NetNES1.1-predicted NESs (http://www.cbs.dtu.dk/services/NetNES/; la Cour et al., 2004), we first established the common NES consensus. As previously proposed (Fu et al., 2011), a consensus sequence [LIVFM]-x-(2,3)-[LIVFM]-x(2,3)-[LIVFM]-x-[LIVFM] with positions 6, 9, and 11 being predominated by leucine was predicted (Figure 4a). All the tested CRM1 export cargoes, including the ones investigated here, readily conformed to this consensus. Interestingly, however, sequence alignment of putative NESs from extracellular signal-regulated kinase 1/2 (ERK1/2), as predicted by NetNES1.1, with the previously established NES sequences, exhibited a sharp departure from this consensus (Figure 4b). Notably, the first two hydrophobic residues (positions 1 and 6) were separated by four amino acids, an observation lacking in any of the examined NESs. Furthermore, the classic C-terminal consensus ([LIVFM]-x-[LIVFM]) of two hydrophobic residues interrupted by a non-hydrophobic residue was distorted by the presence of three consecutive hydrophobic amino acids [LIF]. Based on the previous suggestion by Englmeier et al. (2001), these analyses suggested ERK1/2 as a bearer of a weak NES and, thus, potential seeker of RanBP3 cofactor functionality for their CRM1-mediated nuclear exit (Figure 4c). Indeed, we observed the requirement of RanBP3 in ERK1/2 nuclear export (Figure 4d and e). Curiously, two other CRM1 cofactors (Nup98 and Nxt1) showed complete dispensability for ERK1/2 nuclear export (Figure 4d and e). Unchanged ERK1/2 levels refuted possible protein expression changes as a confounding factor in the immunofluorescence-based localization studies (Supplementary Figure S6 online). Furthermore, an interaction between RanBP3 and ERK1/2 that was immune to Leptomycin B suggested a rather direct mode of interaction (Figure 4f and g). In addition, on RanBP3 silencing, the induction of c-Fos and Cyclin D1 levels (Supplementary Figure S7 online) that is frequently associated with nuclear ERK signaling (Lopez-Bergami et al., 2007) corroborated a heightened ERK nuclear activity and localization.

BAD is a proapoptotic protein that executes its function in the cytoplasmic and mitochondrial compartment. Oncogenic MAPK signaling overcomes this proapoptotic BAD activity through p90RSK1-mediated site-specific (Ser112) BAD phosphorylation (Fang et al., 1999). With RanBP3 silencing rendering significant levels of ERK restricted to the nucleus and ERK being the sole positive modulator of p90RSK1 activity, we predicted a suppression of p(Ser112)-BAD levels on RanBP3 silencing. Indeed, RanBP3 knockdown caused a significant downregulation of inhibitory p(Ser112)-BAD levels (Figure 4h).

Altogether, these data suggest a high degree of RanBP3 dispensability and selectivity in CRM1-mediated nuclear export. These results, while showing RanBP3 requirement in ERK nuclear export, also suggest the mechanistic basis for the apoptotic outcome of RanBP3 ablation through the restoration of proapoptotic dephosphorylated BAD.

MAPK signaling and RanBP3 activity

We analyzed previously generated gene expression data (GEO Series accession no. GSE8401; Xu et al., 2008) from melanocytic nevi ($n = 9$), primary ($n = 31$), and metastatic ($n = 52$) human melanoma tissue samples for *RanBP3* expression levels. No significant change in *RanBP3* transcript levels was observed with the disease progression from nevi to primary and from primary to metastatic stage (Figure 5a). A recent report demonstrating a critical post-translational control of RanBP3 activity through its Ser-58 phosphorylation downstream of ERK and phosphatidylinositol-4,5-bisphosphate 3-kinase signaling (Yoon et al., 2008), together with a frequent constitutive activity of these signaling modules in melanoma, alluded to a post-translational control of RanBP3 activity in melanoma cells. To test this proposition, we subjected 16 melanoma cell lines to a systematic correlation between effective p-RanBP3 and p-ERK1/2 levels. While demonstrating high p-ERK1/2 levels, the majority of cell lines also showed inherently high p-RanBP3 levels (Supplementary Figure S8 online). Among the 16 tested melanoma cell lines, 12 exhibited a positive correlation (Pearson correlation $r = 0.42$) between effective p-ERK and p-RanBP3 (Supplementary Table S2 and Supplemental Materials online), but because of the limited sample size, it did not reach statistical significance ($P > 0.05$). To functionally evaluate a causal relationship between MAPK signaling and RanBP3 phosphorylation, we subjected *NRAS*-mutant Sk-Mel2 and *BRAF*-mutant M14 melanoma cells to MEK inhibition. Notably, two distinct MEK inhibitors (PD98059 and U0126) that downregulated p-ERK levels also compromised RanBP3 phosphorylation (Figure 5b). Providing further support to this signaling nexus, a parallel comparison of BRAF inhibitor-sensitive WM983B and corresponding BRAF inhibitor-resistant clone WM983B-BR showed, in the latter, elevated p-RanBP3 levels that were in line with upregulated p-ERK levels (Figure 5c). Altogether, these results suggested a post-translational (phosphorylation-based) regulation of RanBP3 in melanoma cells, downstream of constitutively hyperactive MEK-ERK signaling.

immunoblotting. (**e**) Top: Immunofluorescent staining for p53 (top panel) in UACC-62 and Sk-Mel5 cells on 72 hours of Ct.siRNA or si-RanBP3#1 treatment. Nuclear staining with TO-PRO (middle panel). Overlay of p53 and nuclear stain (bottom panel). Scale bar = 20 μm. (**e**) Bottom: Indicated melanoma cells were transfected with Ct.siRNA or si-RanBP3#1 for 72 hours followed by the immunoblotting-based assessment of p53 levels. (**f**) Left: Immunofluorescence-based staining for MEK1 in the indicated cell lines on 6 hours of LMB (4 nM) treatment. (**f**) Right: Corresponding quantification. Scale bar = 20 μm. (**g**) Top: Immunofluorescent staining for MEK1 (left panel) in the indicated cell lines on 72 hours' treatment with Ct.siRNA or si-RanBP3 (#1 or #2). Nuclear staining with DAPI (middle panel). Overlay of MEK1 and nuclear stain (right panel). (**g**) Bottom: Corresponding quantification. Scale bar = 20 μm. Ct.siRNA, control small interfering RNA; LMB, Leptomycin B; MEK, MAPK/ERK kinase; RanBP3, ran binding protein 3.

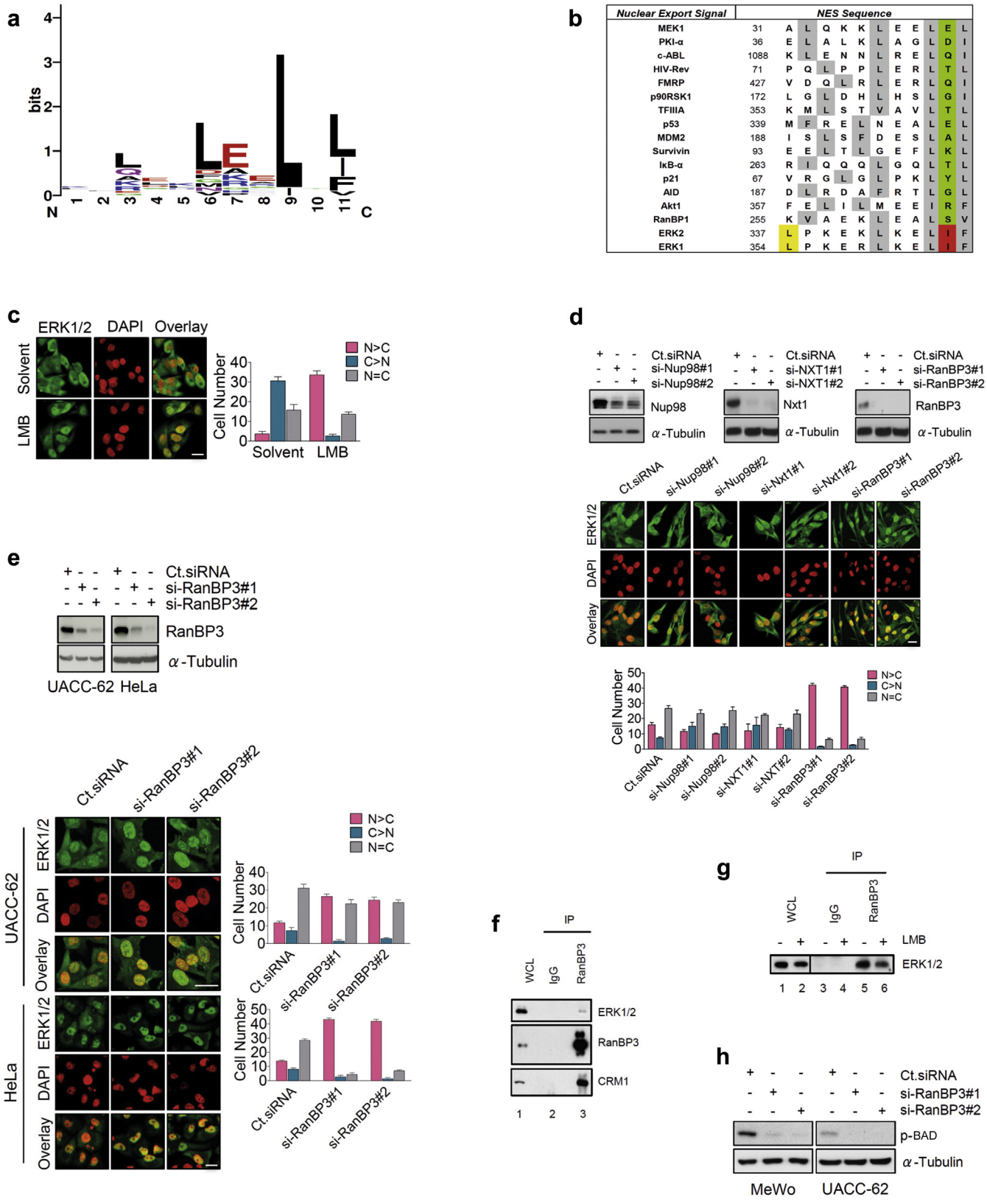

Figure 4. RanBP3 in ERK nuclear export. (**a**) The sequence logo was produced through http://weblogo.berkeley.edu/logo.cgi, utilizing 17 experimentally validated and/or NetNES1.1 (http://www.cbs.dtu.dk/services/NetNES/) predicted NESs. The height of each amino acid one-letter abbreviation reflects the Shannon information content in units of bits. (**b**) The NES sequence alignment of 17 experimentally validated and/or NetNES1.1 predicted NESs. (**c**) Left: Immunofluorescence-based staining for ERK1/2 (left panel) in MeWo cells on 6 hours of LMB (4 nM) treatment. Nuclear staining with DAPI (middle panel). Overlay of ERK1/2 and nuclear stain (right panel). (**c**) Right: Corresponding quantification. Scale bar = 20 μm. (**d**) Top: MeWo cells were treated as indicated for 72 hours followed by the assessment of Nup98 (left), Nxt1 (middle), and RanBP3 (right) levels by immunoblotting. (**d**) Middle: MeWo cells were treated as indicated for 72 hours followed by immunofluorescence-based staining of ERK1/2 (top panel). Nuclear staining with DAPI (middle panel). Overlay of ERK1/2

DISCUSSION

The illustrated ability of RanBP3 ablation to compromise melanoma cell proliferation, regardless of the underlying molecular alterations, could bear major implications from a therapeutic standpoint. Although RanBP3 targeting exhibited objective retardation of melanoma cell proliferation and elicited apoptotic induction, the magnitude of these effects certainly fell short of the ones achieved on CRM1 targeting (Pathria et al., 2012). Given the derailment of multiple cellular pathways consequent to a virtually complete cessation of nuclear export on CRM1 inhibition, these differences are understandable (Pathria et al., 2012). Importantly, the more selective control of nuclear export as executed by RanBP3, while likely circumventing potential long-term toxicity issues of CRM1 targeting, could also present efficient combination approaches to heighten the responses achieved by lone RanBP3 targeting. For example, with the ability to reinstate a TGF-β signaling-associated antiproliferative program, a combination of RanBP3 silencing and TGF-β could translate into heightened clinical benefit.

Although p53 tumor suppressor function is frequently impaired through loss-of-function mutations in various cancers (Olivier et al., 2010), most melanomas do carry an intact p53 (Houben et al., 2011). Although melanomas successfully circumvent some of the p53 cell cycle retarding activities through gene deletions or inactivating mutations in tumor suppressor $p16^{INK4A}$/$p14^{ARF}$ (Chin, 2003; Monzon et al., 1998), the mechanisms that bypass $p21^{Cip1}$ tumor suppressor function are far from clear. The presented data showing the failure of RanBP3 silencing to induce p53 levels, and the induction of $p21^{Cip1}$ in mutant/nonfunctional-p53 harboring MeWo cells, together, refute p53-mediated $p21^{Cip1}$ regulation in the RanBP3 knockdown setting. These data further substantiate the negative regulation of the TGF-β-$p21^{Cip1}$ axis through RanBP3-mediated Smad2/3 nuclear export (Dai et al., 2009). This ability to recruit $p21^{Cip1}$ tumor suppressor function in the absence of functional p53 could carry important therapeutic benefit. Moreover, ERK-activated RanBP3's ability to shuttle Smad2/3 out of the nucleus and, thus, potentially suppress activated MAPK (oncogene)-mediated $p21^{Cip1}$ upregulation and the accompanying senescence program could be a critical step in melanoma cell transformation.

RanBP3 is frequently purported as a CRM1 cofactor. However, to the best of our knowledge, never has this role been experimentally evaluated in the general CRM1-mediated nuclear export. Our data exhibiting absolute dispensability of RanBP3 in the nuclear export of previously established proconsensus NES harboring CRM1 cargoes—MEK, survivin, and p53—weigh into the proposition that RanBP3 cofactor function is dispensable for nuclear export of the proteins that harbor a strong NES (Englmeier et al., 2001). Furthermore, our data suggesting RanBP3 requirement in the nuclear export of a weak NES harboring ERK1/2 further support this idea. In the wake of a previous study by Adachi et al. (2000) suggesting the requirement of MEK in ERK nuclear export, the observed failure of RanBP3 silencing to impair MEK nuclear export hints toward the potential existence of at least two independent pathways of ERK nuclear export: (a) CRM1-mediated MEK-dependent and (b) RanBP3-mediated MEK-independent export. Although our preliminary affinity studies would support these two export models, a previous report by Burack and Shaw (2005) refuting MEK involvement in CRM1-mediated ERK export and a later study by Ebner et al. (2007) suggesting CRM1 dispensability in ERK nuclear export, while alluding to the lack of understanding of ERK export mechanisms, underscore the need of more comprehensive biochemical studies. Furthermore, previous studies witnessing CRM1-independent RanBP3 nuclear export activity (Dai et al., 2009; Hendriksen et al., 2005) and the need to gain greater insights into the antiproliferative mechanisms associated with RanBP3 silencing would argue for a more aggressive analysis of the RanBP3 export repertoire. Interestingly, although a previous report suggested the requirement of RanBP3 function in the maintenance of the Ran cellular distribution in HeLa cells (Yoon et al., 2008), RanBP3 silencing in melanoma cells failed to perturb the Ran distribution (data not shown). This ruled out the contribution of potentially altered Ran localization to RanBP3 knockdown-associated antiproliferative outcome in melanoma cells.

The field of melanoma therapeutics currently focuses on developing new approaches to quantitatively ease the flux through frequently activated MAPK signaling. However, in the wake of highly disparate and continuously evolving mechanisms of MAPK signaling reactivation (Solit and Rosen, 2014), achieving robust quantitative abrogation of this cascade has remained a formidable challenge. Furthermore, in recent years, there has been a consistent realization and appreciation of ERK cellular localization as an important pathogenetic factor, with nuclear ERK signaling and the depletion of cancer-associated cytoplasmic ERK activity as antiproliferative mechanisms (Bollag et al., 2010; Cagnol and Chambard, 2010; Pathria et al., 2012). However, resetting normal ERK nuclear distribution has posed a significant mechanistic challenge, with CRM1 targeting being the only available avenue (Pathria et al., 2012). In light of this information, our data showing RanBP3 requirement in ERK nuclear export suggest a new approach for countering

and nuclear stain (bottom panel). (**d**) Bottom: Corresponding quantification. Scale bar = 20 μm. (**e**) Top: UACC-62 and HeLa cells were treated as indicated for 72 hours followed by the assessment of RanBP3 levels by immunoblotting. Lower left: UACC-62 and HeLa cells were treated as indicated for 72 hours followed by immunofluorescent staining of ERK1/2 (top panel). Nuclear staining with DAPI (middle panel). Overlay of ERK1/2 and nuclear stain (bottom panel). Lower right: Corresponding quantification. Scale bar = 20 μm. (**f**) RanBP3-bound ERK1/2 was immunoprecipitated with an anti-RanBP3 antibody and detected by anti-ERK1/2 antibody. CRM1 staining was included as a positive control. The anti-mouse IgG immunoprecipitates in lane 2 signifies the negative control. (**g**) MeWo cells were treated with methanol 70% (Mock) or LMB (10 nM) for 1 hour. RanBP3-bound ERK1/2 was immunoprecipitated with an anti-RanBP3 antibody and detected by an anti-ERK1/2 antibody. The anti-mouse IgG immunoprecipitates in lanes 3 and 4 signify the negative control. (**h**) MeWo and UACC-62 cells were treated as indicated for 96 hours followed by the assessment of RanBP3 and p(S112)-BAD levels by immunoblotting. DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase1/2; LMB, Leptomycin B; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NES, nuclear export sequence; RanBP3, ran binding protein 3.

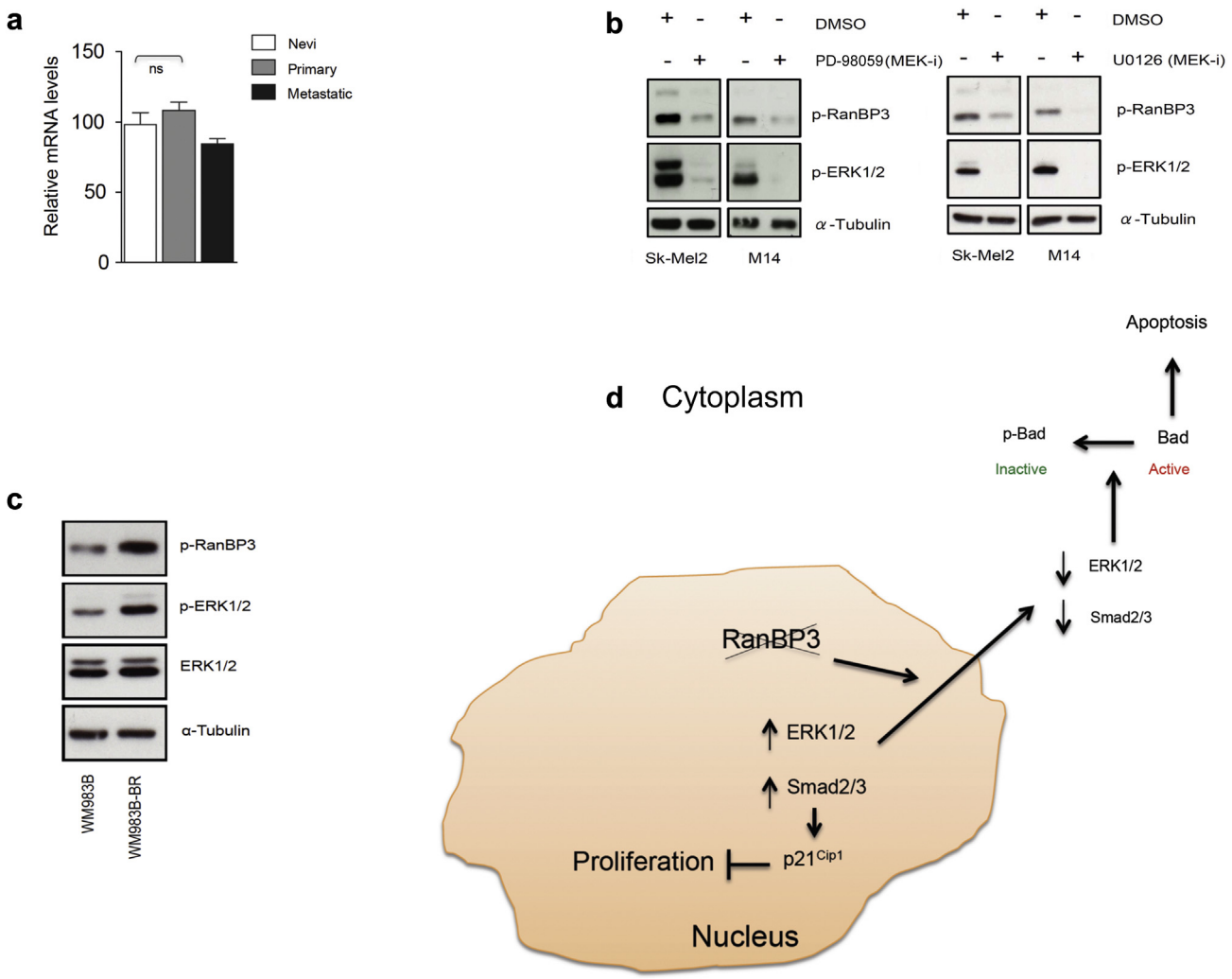

Figure 5. MAPK signaling regulates RanBP3 activity. (**a**) Relative mRNA expression levels (*y*-axis) of *RanBP3* in melanocytic nevi ($n = 9$), primary human melanoma ($n = 31$), and human melanoma metastases ($n = 52$). (**b**) Sk-Mel2 and M14 cells were treated with MEK inhibitor, PD-98059 (10 μM; left) or U0126 (1 μM; right) for 6 hours followed by the immunoblotting-based assessment of the indicated molecules. (**c**) BRAF inhibitor-sensitive WM983B melanoma cells and their BRAF inhibitor-resistant counterpart WM983B-BR were cultured in RPMI + 10% FCS and RPMI + 10% FCS + 1 μM PLX-4032, respectively. At 70% to 80% confluence, the cells were harvested and the lysates were probed for the indicated proteins by immunoblotting. (**d**) A working model for RanBP3 knockdown-mediated suppression of melanoma cell proliferation and apoptosis. Compromising RanBP3 function blocks Smad2/3 and ERK1/2 nuclear export. Nuclear retention of Smad2/3 restores transcriptional regulation of $p21^{Cip1}$ that in turn suppresses melanoma cell proliferation. Depleted ERK1/2 levels in the cytoplasm relieve the inhibitory phosphorylation of BAD, thus restoring the latter's proapoptotic activity. ERK1/2, extracellular signal-regulated kinase1/2; FCS, fetal calf serum; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; ns, nonsignificant (*t*-test); RanBP3, ran binding protein 3.

ERK-associated protumorigenic activities without the constraints of its quantitative easing and the associated pathway reactivation. However, contingent upon RanBP3's greater involvement in the nuclear export process, developing more specific RanBP3-ERK interference strategies could be highly rewarding. Analyzing recombinant fragments corresponding to distinct interaction domains of RanBP3 (Lindsay et al., 2001) in affinity studies and in vitro export assays could help ascertain the regions of ERK interaction. Subsequent peptide competition strategies could prove instrumental in specifically overcoming the RanBP3-ERK interaction.

In conclusion, the findings of this study propose RanBP3 as a downstream effector of MAPK signaling that selectively participates in CRM1-mediated nuclear export to regulate melanoma cell proliferation (Figure 5d). This study provides a basis for similar interrogations of RanBP3 function in other cancers and argues for its further development as melanoma therapeutics.

MATERIALS AND METHODS

Cell culture and reagents

All cell lines were cultured in RPMI (Invitrogen, Carlsbad, CA), supplemented with 10% FCS (Invitrogen). Also see Supplemental Materials online.

Gene expression profiling

Tissue sampling and gene expression profiling were previously performed using the Affymetrix U133A microarray platform as described earlier (Xu et al., 2008). The data have been deposited in the National Center for Biotechnology Information GEO (Clark et al., 2000) and are accessible through GEO Series accession no. GSE8401.

Cell proliferation

The absolute cell number (final cell count) and the increase in the absolute cell number (final cell count − starting cell count) were determined by CASY counter (Roche Innovatis AG, Bielefeld, Germany).

RNA interference

siRNA transfections were performed employing Lipofectamine 2000 transfection reagent (Invitrogen, Vienna, Austria) per the manufacturer's instructions.

β-Galactosidase assay

The β-galactosidase (senescence) assay was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA).

AnnexinV and/or propidium iodide and caspase inhibition assays

AnnexinV/propidium iodide-based apoptosis detection and quantification was performed as previously described (Jalili et al., 2011). For evaluating the effect of pan-caspase inhibitor, melanoma cells were pretreated with Z-VAD-FMK (40 μM; BD Biosciences, Schwechat, Austria) for 2 hours followed by treatment with control siRNA or si-RanBP3#1. 96 hours after siRNA treatment, cells were analyzed for apoptosis, as described above.

Immunoblotting

Western blotting was performed as previously described (Jalili et al., 2011). α-Tubulin staining was used as a control for equal sample loading. See Supplementary Materials for antibodies, densitometry, and the calculation of the Pearson correlation.

Immunofluorescence

Immunofluorescence studies were performed as described previously (Pathria et al., 2012). Images were acquired and analyzed using the Zen 2008 (version 5.0) software on an Axiovert 200 m inverted microscope (Zeiss, Oberkochen, Germany).

Sequence alignment and sequence logos

The 17 experimentally validated and/or NetNES1.1-predicted (http://www.cbs.dtu.dk/services/NetNES/; la Cour et al., 2004) NESs were aligned as described previously (Henderson and Eleftheriou, 2000). The sequence logo was produced through http://weblogo.berkeley.edu/logo.cgi, utilizing the 17 experimentally validated and/or NetNES1.1-predicted NESs. The height of each amino acid one-letter abbreviation reflects the Shannon information content in units of bits (Schneider and Stephens, 1990).

Statistical analysis

Graphpad prism software 5.0 (Graphpad, La Jolla, CA; http://www.graphpad.com) was used to perform statistical analysis by performing an unpaired *t*-test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.jidonline.org, and at http://dx.doi.org/10.1038/JID.2015.401.

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